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(54) Title: NUCLEIC ACID ENCODING RATE AGOUTI RELATED PROTEIN

(57) Abstract

This invention concerns nucleic acid encoding for rat agouti related protein. Agouti related protein is a neuropeptide that may play a role in the central regulation of feeding behavior and energy metabolism via interactions with the melanocortin pathways. This invention also relates to assays utilizing the novel nucleic acid of this invention.

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TITLE OF THE INVENTION

NUCLEIC ACID ENCODING RAT AGOUTI RELATED PROTEIN

FIELD OF THE INVENTION

5 This invention relates to nucleic acids encoding for rat agouti related protein. Agouti related protein is a neuropeptide that may play a role in the central regulation of feeding behavior and energy metabolism via interactions with the melanocortin pathways.

10 BACKGROUND OF THE INVENTION

While normally related to the production of black or yellow pigment in the hair, ubiquitous expression of agouti protein has been implicated in the occurrence of obesity and diabetes in yellow obese AY mice. Agouti related protein (AGRP; also known as ART and AGRT), a
15 recently identified neuropeptide, shares some sequence homology with agouti protein and may play a role in the central regulation of feeding behavior and energy metabolism via interactions with the melanocortin pathways in a manner similar to agouti protein. When tested to see whether this homolog caused the onset of the same symptoms, it was
20 observed that the overexpression of AGRT correlated with weight gain; see Graham et al., 1997 *Nature Genetics* 17:273-274 and Ollmann et al., 1997 *Science* 278:135-138. Moreover, AGRP mRNA is up-regulated in leptin-function deficient animals such as *ob/ob* and *db/db* mice (Shutter et al., *Genes Dev.* 11, 593,602, 1997).

25 The DNA sequence of AGRP has been reported for human and mouse; Shutter et al., 1997 *Genes Dev.* 11:593-602, Ollmann et al., 1997 *Science* 278:135-138. The human gene for ART is a 132-amino acid protein that is 25% identical to human agouti protein and is expressed primarily in the adrenal gland, subthalamic nucleus, and
30 hypothalamus with a lower level of expression occurring in testis, lung, and kidney. The murine homolog of ART encodes a 131-amino acid protein that shares 81% amino acid identity to human ART with localized expression in the arcuate nucleus of the hypothalamus, the median eminence, and the adrenal medulla.

Attempts at detecting *in situ* hybridization signals of AGRP mRNA in rat brain with probes based on mouse AGRP sequences have proved unsuccessful. Since the rat is the primary animal model in the field of feeding regulation research, it is critical to characterize the 5 function of AGRP in rats with the AGRP sequence from the same species. It is, thus, an object of the present invention to provide a compound that can recapitulate these same symptoms in rats so as to further our current knowledge in the field of feeding regulation. Such a compound could be used for developing tools to characterize the 10 biological function of this neuropeptide, and to discover new drugs which modulate AGRP and melanocortin functions.

SUMMARY OF THE INVENTION

This invention relates to isolated nucleic acids encoding rat 15 agouti related protein. Preferred embodiments include nucleic acid of the sequence set forth in SEQ ID NO:1 and nucleic acids which are complementary to it. This invention also relates to the protein encoded thereby (rat AGRP), vectors comprising said nucleic acid, and host cells comprising said vectors. Lastly, the present invention relates to 20 processes utilizing the nucleic acids or protein of the present invention, such as the generation of antibodies to rat AGRP through the injection of fragments or full length rat AGRP into rabbits and recovery of the antibodies therefrom.

25 BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts the DNA sequence of rat AGRP (SEQ ID NO:1).

FIGURE 2 depicts the amino acid sequence of rat AGRP (SEQ ID NO:2).

30

DETAILED DESCRIPTION OF THE INVENTION

As used throughout the specification and claims, the following definitions apply:

“AGRP” means agouti related protein. Throughout the 35 specification, AGRP, ART and AGRT are referred to interchangeably.

"ART" or "AGRT" means agouti related transcript. This term is used to describe a nucleic acid molecule or fragment thereof that (a) comprises the nucleotide sequence as set forth in SEQ ID NO:1; (b) comprises a nucleic acid sequence encoding a polypeptide that is at least 5 70 percent identical, preferably at least 80 percent identical and most preferably at least 90 percent identical to the polypeptide encoded by SEQ ID NO:1; (c) is a naturally occurring allelic variant of (a) or (b); (d) is a nucleic acid variant of (a)-(c); and/or (e) is complementary to (a)-(d).

"ART protein" or "ART polypeptide" or "rat AGRP" as used 10 herein refers to any protein or polypeptide having the properties characteristic of ART (i.e., the capability of modulating the signaling activity of a melanocortin receptor, the capability of modulating intracellular cyclic AMP (cAMP) levels, and/or the capability of modulating lipid metabolism). The ART polypeptide may or may not 15 have an amino terminal methionine. By way of illustration, ART protein or ART polypeptide includes an amino acid sequence encoded by the nucleic acid molecule set forth in any of items (a) or (e) above and peptide or polypeptide fragments derived therefrom, the amino acid sequence set forth in SEQ ID NO:2 and/or chemically modified 20 derivatives as well as nucleic acid and/or amino acid sequence variants thereof.

"ART fragment" or "fragment of agouti-related protein" refers to a peptide or polypeptide that is less than the full length amino acid sequence of naturally occurring ART protein but has substantially 25 the same biological activity as ART polypeptide or ART protein described above. Such a fragment may be truncated at the amino terminus, the carboxy terminus, and/or internally, and may be chemically modified. Preferably, the ART fragment will be a carboxy terminal fragment which retains at least all 10 C-terminal cysteine residues. Such ART 30 fragments may be prepared with or without an amino terminal methionine.

"ART derivative" or "ART variant" refers to an ART polypeptide or ART protein that has 1) been chemically modified, as for example, by addition of polyethylene glycol or other compound, and/or 2)

contains one or more nucleic acid or amino acid sequence substitutions, deletions, and/or insertions.

“Biologically active polypeptide” and “biologically active fragment” refer to a peptide or polypeptide that has ART activity (i.e., is 5 capable of modulating the signaling activity of a melanocortin receptor, is capable of modulating intracellular cAMP levels, and/or is capable of modulating lipid metabolism).

“Stringent conditions” means approximately 35°C to 65°C in a salt solution of approximately 0.9 molar NaCl. Stringency may also be 10 governed by such reaction parameters as the concentration and type of ionic species present in the hybridization solution, the types and concentrations of denaturing agents present, and the temperature of hybridization. Generally as hybridization conditions become more stringent, longer probes are preferred if stable hybrids are to be formed. 15 As a rule, the stringency of the conditions under which a hybridization is to take place will dictate certain characteristics of the preferred probes to be employed. Such relationships are well understood and can be readily manipulated by those skilled in the art.

The present invention relates to newly identified nucleic 20 acids encoding rat agouti related protein (AGRP), and more preferably nucleic acid comprising the sequence set forth in SEQ ID NO:1. In comparison to the human and mouse AGRP, the rat sequence exhibits marked diversity including most significantly a deletion of 4 and 3 amino acid residues, respectively, in the N-terminal region. This 25 structural difference between species may explain failed attempts to detect *in situ* hybridization signals of AGRP mRNA in rat brain by using probes based on mouse AGRP sequences. The present invention also relates to isolated rat AGRP and, more preferably, rat AGRP comprising the sequence set forth in SEQ ID NO:2.

Nucleic acid in accordance with the present invention, and 30 preferably cDNA, can be isolated by methods well known in the art including, without limitation, polymerase chain reaction (PCR), as well as cDNA and/or genomic library screening with suitable probes (usually oligonucleotides and/or antibodies for cDNA libraries, and 35 oligonucleotides or cDNA sequences for genomic libraries). Some of

these methods as well as other methods useful for molecular cloning are set forth by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). The nucleic acid may be any nucleic acid which can encode a protein 5 such as genomic DNA, cDNA or any of the various forms of RNA.

Polypeptides constitute yet another aspect of the invention and may be naturally occurring full length polypeptides, or truncated polypeptides or peptides (i.e., "fragments"). The polypeptides or fragments may be chemically modified, i.e., glycosylated, 10 phosphorylated, and/or linked to a polymer and they may have an amino terminal methionine. In addition, the polypeptides or fragments may be variants of the naturally occurring ART polypeptide (i.e., may contain one or more amino acid deletions, insertions, and/or substitutions as compared with naturally occurring ART). The polypeptide of Figure 2 is 15 129 amino acids long with an open reading frame of 387 basepairs, and exhibits 93.8% homology to mouse AGRP at the amino acid level and 78.3% homology to human AGRP at the amino acid level.

The full length AGRP polypeptide or fragment thereof can be prepared using well known recombinant DNA technology methods 20 such as those set forth in Sambrook et al., *supra*, and/or Ausubel et al., eds., *Current Protocols in Molecular Biology*, Green Publishers Inc. and Wiley and Sons, NY (1994). A gene or cDNA encoding the ART protein or fragment thereof may be obtained for example by screening a genomic or cDNA library, or by PCR amplification. Alternatively, a gene 25 encoding the ART polypeptide or fragment may be prepared by chemical synthesis using methods well known to the skilled artisan such as those described by Engels et al., 1989 *Angew. Chem. Intl. Ed.* 28:716-734. These methods include, *inter alia*, the phosphotriester, phosphoramidite, and H-phosphonate methods for nucleic acid 30 synthesis. A preferred method for such chemical synthesis is polymer-supported synthesis using standard phosphoramidite chemistry.

Typically, the DNA encoding the ART polypeptide will be several hundred nucleotides in length. The cDNA of Figure 1 is 470 basepairs in length, exhibiting 94.6% homology to mouse AGRP at the 35 nucleotide level and 82.9% homology to human AGRP at the nucleotide

level. Nucleic acids larger than about 100 nucleotides can be synthesized as several fragments using these methods. The fragments can then be ligated together to form the full length ART polypeptide. Usually, the DNA fragment encoding the amino terminus of the polypeptide will have

5 an ATG, which encodes a methionine residue. This methionine may or may not be present on the mature form of the ART polypeptide, depending on whether the polypeptide produced in the host cell is secreted from that cell.

Nucleic acid and/or amino acid variants of naturally occurring ART are also encompassed within the scope of this invention. Nucleic acid variants (wherein one or more nucleotides differ from the wild-type or naturally occurring ART) may be produced using site directed mutagenesis or PCR amplification where the primer(s) have desired point mutations; see Sambrook et al., *supra*, and Ausubel et al., 15 *supra*, for descriptions of mutagenesis techniques. Chemical synthesis using methods described by Engels et al., *supra*, may also be used to prepare such variants. Other methods known to the skilled artisan may be used as well.

The ART gene or cDNA can be inserted into an appropriate. 20 expression vector for expression in a host cell; such vector comprising the nucleic acid of the instant invention constituting one further aspect of the invention. The vector may be any known vector, including plasmids, cosmids and viral vectors which can function in a chosen host cell. Preferred vectors for practicing this invention are those which are 25 compatible with bacterial, insect, and mammalian host cells. Such vectors include, *inter alia*, pcDNA3 (Invitrogen Company, San Diego, CA), pBluescriptII (Stratagene Company, LaJolla, CA), and BlueBacII (Invitrogen). The vector utilized should be selected to be functional in the particular host cell employed (i.e., the vector is compatible with the host 30 cell machinery such that amplification of the ART gene and/or expression of the gene can occur). The AGRP polypeptide or fragment thereof may be amplified/expressed in prokaryotic (such as *E. coli*) or eukaryotic (including yeast, insect and mammalian) host cells.

Typically, the vectors used in any of the host cells will 35 contain a 5' flanking sequence (also referred to as a "promoter"),

whether it be a native (i.e., from the same species and/or strain as the host cell), heterologous (i.e., from a different species and/or strain), or a hybrid thereof. Optionally, the vectors may also contain other expression-control elements, such as enhancers and sequences which

5 assist the host in expressing the peptide, such as an origin of replication element, a transcriptional termination element, a complete intron sequence containing a donor and acceptor splice site, a signal peptide sequence, a ribosome binding site element, a polyadenylation sequence, a polylinker region for inserting the nucleic acid encoding the

10 polypeptide to be expressed, and/or a selectable marker element. Where such elements are not already present in the vector to be used, they may be individually obtained and ligated into the vector. Methods used for obtaining these elements are well known to the artisan of ordinary skill in the art.

15 The nucleic acid may then be transfected into a selected cell line for expression and evaluation; the host having been transfected with a vector of the instant invention constituting another aspect of the present invention. Transfection can be accomplished using any known method, including without limitation, the calcium phosphate procedure, 20 electroporation, viral infection (e.g., via the use of retroviruses), lipofection, DEAE-dextran, or microinjection. The transfection method used will depend in part on the cell type being transfected. For bacterial cells, electroporation is generally preferred. For mammalian cells, transfection can be accomplished using electroporation, or alternatively, 25 the DEAE-dextran method as described in Section 9.2 of Ausubel et al., *Current Protocols in Molecular Biology*, 1987.

The host cell may be any cell or cell line which is conveniently cultured. In general, a preferred host cell will be eukaryotic, preferably mammalian and, most preferably, selected from 30 the group consisting of Chinese Hamster Ovary cells, HeLa cells, COS-1 and COS-7 monkey cells, melanoma cell lines (such as Bowes cells), mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines, and the CV-1 cell line. Normal diploid cells, cell strains derived from *in vitro* culture of primary tissue, as well

as primary explants (including relatively undifferentiated cells such as hematopoietic stem cells) are also suitable.

Other suitable host cells are prokaryotic cells, such as bacterial cells (in particular, the various strains of *E. coli*, for example 5 HB101, DH5 α , DH10, and MC1061).

Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention.

10 The host cell, when cultured under appropriate conditions, can synthesize AGRP protein which can subsequently be collected from the culture medium (if the host cell secretes it into the medium) or directly from the host cell producing it (if it is not secreted). After collection, the AGRP protein can be purified using methods such as 15 molecular sieve chromatography, affinity chromatography, and the like.

The present invention, thus, includes a process for producing rat agouti related protein comprising constructing a vector comprising the nucleic acid of the present invention, transforming a host cell with said vector, culturing said transformed cell and 20 recovering said protein from the culture.

The present invention also includes a process for producing anti-rat agouti related protein antibodies wherein (i) the full length agouti related protein or fragment thereof is injected into a rabbit or other animal recognized as optimal for generating antibodies and (ii) 25 antibodies specific for rat AGRP are recovered. Methods in accordance with this aspect of the invention are well known to one of ordinary skill in the art.

The following non-limiting Examples are presented to better illustrate the present invention.

30

EXAMPLE 1

RNA Preparation

Nine fatty Zucker rats (male, 600-800 grams, 6 months old) 35 were decapitated under carbon dioxide (CO₂) anesthesia. The

hypothalami were quickly removed and frozen in liquid nitrogen. Total RNA was prepared from the hypothalamic tissues using the ULTRSPECTM RNA isolation kit (Bioteclx Laboratories, Inc., Houston, TX) following the manufacturer's protocol.

5

EXAMPLE 2

RT-PCR

A CDNA TEMPLATE FOR PCR REACTIONS WAS
10 SYNTHESIZED USING 1 μ G OF THE RAT HYPOTHALAMIC RNA
AND BOTH RANDOM AND OLIGO DT PRIMING WITH THE
ADVANTAGE RT-FOR-PCR KIT (CLONTECH, PALO ALTO, CA),
FOLLOWING THE MANUFACTURER'S PROTOCOL. THE FIRST
ROUND OF PCR WAS CARRIED OUT BY MIXING 2 μ L 10 MM DNTP,
15 10 μ L 10X PCR BUFFER, 4 μ L 50 MM MGCL₂, 10 μ L OF REVERSE
TRANSCRIBED CDNA (AN EQUAL MIXTURE OF RANDOM AND
OLIGO DT PRIMED PRODUCTS), AND 5 μ L EACH OF 20 MM PCR
PRIMER #290 (5'- TAGATGGATCCATGCTGACCGCAATG-3'; SEQ ID
NO:3) AND #291 (5'- AGTCAGAATTCTAGGTGCGGCTGCA-3'; SEQ ID
20 NO:4), AND DEIONIZED WATER TO A TOTAL VOLUME OF 100 μ L.
AFTER BEING HEATED AT 95 $^{\circ}$ C FOR 5 MIN., THE SAMPLE TUBE
WAS PLACED ON ICE, THEN 2.5 U OF TAQ POLYMERASE WAS
ADDED TO THE TUBE FOLLOWED BY THE ADDITION OF 50 μ L OF
MINERAL OIL. THE PCR CONDITIONS WERE AS FOLLOWS: 94 $^{\circ}$ C X
25 1 MIN. FOLLOWED BY AN ANNEALING STEP OF 42 $^{\circ}$ C X 1.5 MIN.,
FOLLOWED BY 72 $^{\circ}$ C X 2 MIN., AND THE WHOLE CYCLE WAS
REPEATED 20 TIMES. THE SECOND ROUND (NESTED) PCR WAS
CARRIED OUT BY MIXING 2 μ L 10 MM DNTP, 10 μ L 10X PCR
BUFFER, 4 μ L 50 MM MGCL₂, 1 μ L OF FIRST ROUND PCR PRODUCT
30 (AS TEMPLATE), AND 5 μ L EACH OF 20 MM PCR PRIMER #292 (5'-
CTGGCACTGCCCTGCCAC -3'; SEQ ID NO:5) AND #294 (5'-
GCACATGGGTACAGCA -3'; SEQ ID NO:6), AND DEIONIZED
WATER TO A TOTAL VOLUME OF 100 μ L. THE CYCLING
CONDITIONS WERE THE SAME AS THE FIRST ROUND PCR EXCEPT
35 THAT ANNEALING TEMPERATURE WAS CHANGED TO 45 $^{\circ}$ C AND

THE CYCLES WERE REPEATED 25 TIME. THE PCR PRODUCT OF
ABOUT 200 BP WAS PURIFIED WITH QIAQUICK PCR
PURIFICATION KIT (QIAGEN, VALENCIA, CA), AND VERIFIED
TO BE RAT AGRP BY DNA SEQUENCING.

5

EXAMPLE 3

Screening of cDNA library

The above-mentioned 200 bp PCR products of rat AGRP
10 were purified on a 1% agarose gel followed by Qiaex II Kit (Qiagen,
Valencia, CA) and labeled by random-priming with [α -32P] dCTP using
Redi-Prime kit (Amersham, Arlington Heights, IL) to a specific activity
of 2×10^8 dpm/ μ g DNA. This radiolabeled probe was used to screen a
cDNA library of rat hypothalamus (Howard et al., FEBS Lett. 405, 285-
15 290, 1997). The library (about 700,000 cfu) was plated onto LB/ampicillin
plates overlaid with nylon filters. After overnight growing at 37°C, the
replica filters were made, processed and hybridized at 42°C for 18 hours
in 6 x SSC buffer containing 50% formamide, 5 X Denhardt's solution,
0.1% SDS, 20 μ g/ml sheared salmon-sperm DNA with the 2×10^6 cpm/ml
20 32P-labeled probes. The filters were washed in 0.1 X SSC, 0.1% SDS at
65°C, and exposed to X-ray films for 48 hours. Two independent clones
were identified. DNA was prepared from these clones using WIZARD
miniprep kit (Promega, Madison, WI), and subjected to semi-automated
sequence analysis using the PRISM Dye Deoxy terminator cycle
25 sequencing kit (Applied Biosystems, Foster City, CA) on an ABI 377
sequencer.

WHAT IS CLAIMED IS:

1. Isolated nucleic acid encoding rat agouti related protein.
- 5 2. Nucleic acid in accordance with claim 1 wherein the sequence comprises that set forth in SEQ ID NO:1.
- 10 3. Nucleic acid in accordance with claim 2 which is cDNA.
4. Isolated rat agouti related protein.
- 15 5. Isolated rat agouti related protein in accordance with claim 4 wherein the sequence comprises that set forth in SEQ ID NO:2.
6. A vector comprising the nucleic acid of claim 2.
7. A vector according to claim 6 which is a viral vector.
- 20 8. A host cell comprising a vector in accordance with claim 6.
9. A host cell according to claim 8 which is a mammalian cell.
- 25 10. A host cell according to claim 8 which is *E. coli*.
11. A process for producing rat agouti related protein comprising constructing a vector comprising the nucleic acid of claim 2, transforming a host cell with said vector, culturing said transformed cell and recovering said protein.
- 30 35 12. The process of claim 11 wherein the host cell is a eukaryotic cell.

1/2

cDNA sequence of rat AGRP

1 CAAAGGCCAT GCTGACTGCA ATGTTGCTGA GCTGTGTCT GCTGCTGGCA
51 CTGCCTCCA CATTGGGGGT CCATATGGGC GTGGCACCAC TGAAGGGCAT
101 CAGAAGGTCT GACCAGGCC C TGTTCCCAGA GTTCTCAGGT CTAAGTCTGA
151 AGAAGACAGC AGCAGACCGA GCAGAAGATG TTCTGCTGCA GAAGGCAGAA
201 GCTTTGGCAG AGGTGCTAGA TCCACAGAAC CGCGAGTCTC GTTCTCCGCG
251 TCGCTGTGTA AGGCTGCACG AGTCCTGCTT GGGACAACAG GTACCTTGCT
301 GCGACCTCTC CGCCACGTGC TACTGCCGCT TCTTCAATAC CTTTGCTAC
351 TGCCGCAAGC TAGGTACTGG CACCACGAAC CTCTGCAGCC GCCCCTAGCC
401 AACGGATGTT GGGCAAAGGC AGGGGACGCG AATAAACGAT GGGACTAACT
451 CTAAAAAAA AAAAAAAA

FIG.1

2/2

Protein sequence of rat AGRP

1 MLTAMLLSCV LLLALPPTLG VHMGVAPLKG IRRSDQALFP EFSGLSLKKT
51 AADRAEDVLL QKAEALAEVL DPQNRESRSP RRCVRLHESC LGQQVPCCDL
101 CATCYCRFFN TFCYCRKLGT GTTNLCSR*P

FIG.2

SEQUENCE LISTING

<110> Merck & Co., Inc.

<120> NUCLEIC ACID ENCODING RAT AGOUTI RELATED PROTEIN

<130> 20208 PCT

<150> 60/083,549

<151> 1998-04-29

<160> 6

<170> FastSEQ for Windows Version 3.0

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<211> 470

<212> DNA

<213> *rattus horvegicus*

<400> 1

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tgttccca	aga	ttctcaagg	ctaagtctga	agaagacagc	agcagaccga	180
ttctgtgc	gaaggcagaa	gttggcag	aggtgctaga	tccacagaac	cgcgagtctc	240
ttctcccg	tcgctgtgt	aggctgcac	agtccgtctt	gggacaacag	gtaccttgct	300
gcgacctgt	cgccacgtgc	tactgcccgt	tcttcaatac	cttttgctac	tggcgaagc	360
taggtactgg	caccacgaac	ctctgcagcc	gcccctagcc	aacggatgtt	ggcacaaggc	420
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<210> 2

<211> 129

<212> PRT

<213> *rattus horvegicus*

<400> 2

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1				5				10					15		
Pro	Thr	Leu	Gly	Val	His	Met	Gly	Val	Ala	Pro	Leu	Lys	Gly	Ile	Arg
				20				25				30			
Arg	Ser	Asp	Gln	Ala	Leu	Phe	Pro	Glu	Phe	Ser	Gly	Leu	Ser	Leu	Lys
				35				40			45				
Lys	Thr	Ala	Ala	Asp	Arg	Ala	Glu	Asp	Val	Leu	Leu	Gln	Lys	Ala	Glu
				50				55			60				
Ala	Leu	Ala	Glu	Val	Leu	Asp	Pro	Gln	Asn	Arg	Glu	Ser	Arg	Ser	Pro
				65				70			75			80	
Arg	Arg	Cys	Val	Arg	Leu	His	Glu	Ser	Cys	Leu	Gly	Gln	Gln	Val	Pro
				85				90			95				
Cys	Cys	Asp	Leu	Cys	Ala	Thr	Cys	Tyr	Cys	Arg	Phe	Phe	Asn	Thr	Phe
				100				105			110				
Cys	Tyr	Cys	Arg	Lys	Leu	Gly	Thr	Gly	Thr	Thr	Asn	Leu	Cys	Ser	Arg
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Pro															

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26

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17